Development of a Double Antigen Tetanus ELISA for Use in Horses

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Abstract

Horses are commonly vaccinated against tetanus due to the high mortality rate associated with the toxin from the ubiquitous bacterium, *Clostridium tetani.* The immunogenicity of any vaccine is dependent upon the adjuvant used and the horse's previous vaccination history. Inconsistent vaccination practices can lead to lack of protection in case of a challenge infection; however, it is hard to quantify the level of protective immunity. Creating an enzyme-linked immunosorbent assay (ELISA) that will accurately detect antibody levels below 0.10 IU/mL will provide researchers with a cost-effective and timely method to precisely measure tetanus-specific antibody titers. A double antigen ELISA method creates a sandwich of the tetanus specific antigens around the sample antibody. The secondary antigen added is labeled with a biotin marker allowing for a colored conjugate to react. This creates a gradation of a colored reaction, allowing for the ELISA plate reader to determine the antibody titers. This is applicable in both commercial and educational settings that are investigating immune responses and vaccine efficacy. The results of this study indicate that the biotin labeled secondary antigen and the tetanus IgG in the serum may not be properly matching, leading to inconsistent IgG readings. One possible explanation for these inconsistent results is that the blocking solution may not have been added in the correct volume, giving inaccurate IgG readings. Suggested improvements to the protocol used in this experiment include plating known amounts of serum to the plate first to then bind the primary and labeled secondary antigen.

Introduction

Tetanus is an infectious disease that is commonly vaccinated against due to its high mortality rate and difficult treatment in horses. Tetanus is caused by toxins produced by the bacteria *Clostridium Tetani* that is commonly found in intestinal tracts of many animals as well as in the soil. Almost all mammals are susceptible to tetanus infection; however, horses appear to be the most sensitive, other than humans (Merck Veterinary Manual, 2011). Vaccination against tetanus is an important part of equine health management due to the opportunity for chance infection throughout a horse's life (Leifman, 1980; Holmes, 2006).

These bacteria typically enter the body through an open sore, puncture wounds, or exposed tissue and then release tetanus neurotoxin. The *Clostridium Tetani* bacteria grow spores in an anaerobic environment until it goes through autolysis and releases toxin (Merck Veterinary Manual, 2011). This neurotoxin is absorbed by motor neurons and taken to the central nervous system, where it causes descending tetanus. This may trigger the muscle spasms associated with tetanus, and in humans, 'lock jaw' is a common symptom. These spasms may be so severe that they cause bone fractures. Spasms affecting the respiratory system can cause respiratory failure by affecting the larynx and diaphragm. When the toxin affects the autonomic nervous system, the resulting symptoms include cardiac arrhythmias, tachycardia and hypertension (American Equine Practitioners, 2008).

Tetanus is clinically diagnosed through toxin levels in the serum or symptoms such as colic, stiffness, spasms, and third eyelid protrusion (Merck Veterinary Manual, 2011). Treatment is limited to giving large doses of tetanus antitoxin directly to the animal, mixed with small amount of toxoid, or killed toxin. This toxoid stimulates the immune system to

create antitoxin to fight the infection. Horses that contract tetanus with no history of tetanus vaccinations or inoculated with improper vaccination practices have high mortality rates. Penicillin is also administered to help fight the bacterial spores and surgery can be a treatment options for some cases (Rosskopf et al., 2005). It is possible to recover from a tetanus infection if caught early; however, mortality rates are very high.

The high mortality rate associated with tetanus and the success of tetanus vaccinations in protecting from infection leads many horse owners to choose vaccination against this pathogen. The most common form of tetanus vaccination used in the United States today is an inactivated tetanus toxoid (Merck Veterinary Manual, 2011). Active vaccinations consist of just toxoid, while passive vaccinations consist of anti-toxin only. This is considered passive due to the lack of humoral response elicited from anti-toxin. Anti-toxin does give immediate protection against infection however it is quickly eliminated from the body (Leifman, 1981). Active immunization using toxoid creates an immune response in seven to fourteen days, and there is typically a strong immunogenic effect. This protection can be lasting and very effective against future challenge infections due to the circulating anti-toxin available (Heldens et al., 2010).

Vaccination schedules vary according to the specific manufacturer and vaccine, however it typically consists of an original injection, followed by a booster 4 to 6 weeks later. This is followed up with a yearly revaccination. Some require another booster around six months following the original vaccination in order to ensure protection for the first year (Heldens et al., 2010). Re-vaccination is commonly recommended following injury, and can be done in conjunction with injection of antitoxin or passive vaccination (Merck Veterinary Manual, 2011).

These vaccination schedules are based on research on duration of immunity however every horse responds differently to the vaccines and have varying levels of immune response (Leifman 1980; Holmes, 2006). Vaccine efficacy is measured by immune response. In the example of a tetanus bacterial infection, protection is measured by quantity of antibodies available in the bloodstream. Values greater than 0.10 IU/ml are considered to have minimal protection against infection (Perry et al., 2009). Values greater than 1.0 IU/mL are high titre levels imply a high level of protection. Intermediate levels imply protective status, and can benefit from vaccination. In one study, it was observed that vaccination while tetanus antibody titers were significantly above 1.0 IU/mL increased the titers significantly due to their originally high status (Perry et al., 2009). For example, the vaccinated horse group received a tetanus vaccination at week 0, 4, and 26. Week 6 and 30 recorded the highest levels of tetanus antibodies in the sera. The week 26 booster was able to give immunity until the next vaccination at week 129, however immunity status dropped significantly over the remaining interval till re-inoculation. Over the next year before revaccination, the antibody or antiserum titers can drop significantly and leave the animal susceptible to infection. In another study, it was found that 2 doses of a combined active-passive vaccine was required to ensure twelve months of protection (Liefman, 1981). The researchers noted the production of vaccine and adjuvant can be a significant factor in the immunogenicity. Horses can also react to vaccines differently on an individual basis, with no two vaccines creating the same response. Also, waiting too long between vaccinations can leave the animal susceptible to infection. This highlights the importance of a correct vaccination schedule.

Quantifying immune responses in order to determine protective status can be done in many ways. For tetanus, measuring the specific immunoglobulin G (IgG) levels gives the most accurate answer. *In vivo* methods used to be the most commonly used test, however the high mortality rate for mice and guinea pigs injected with toxin has lead to research for more viable *in vitro* methods (Kristiansen et al., 1997). Some in vitro methods include various ELISA tests including indirect, sandwich and competitive. Indirect ELISA has low accuracy when quantifying low levels of titer and typically reads higher levels (How to Run an ELISA, 2011). Competitive and Sandwich ELISA both require very specific types of antigen and antibody pairings that are not cost effective. ELISA tests are very common tools for calculating antigen and antibody levels and have many different manufacturers. Van Hoeven et al. (2008) found that among three commercially available ELISAs there were differences in antibody levels reported from the same serum. While all were within acceptable limits when related to control, some tests typically ran higher antibody levels compared to the others. Also, imprecision at a low amount can be the difference between protection and possible susceptibility.

Many researchers and vaccine manufacturers are interested in having a more accurate assessment to test vaccine efficacy and duration. Vaccine manufacturers would benefit by having an efficient test of quantify efficacy. Researchers looking into immunity and how the immune response changes in regards to tetanus would have an easier way to quantify immunity without having to buy cost prohibitive ELISA premade kits. This test specifically targets the IgG for tetanus, allowing for further evaluation of humoral immune responses.

Rationale

A simple, cost-effective and accurate test for tetanus antibody titer levels in horse serum would allow a way to quantify immune responses. This test would be useful in both commercial and educational settings that are researching vaccine efficacy, immune responses, or many other physiological aspects. This could also be used in a veterinary setting for the purpose of diagnostics and vaccinations scheduling. The previous research done in this field does not take into account cost and widespread availability. The proposed research will provide insight into whether a double antigen tetanus ELISA is a feasible method to quantify tetanus-specific IgG responses.

Materials and Methods

Six Quarter Horses (1.5 ± 0.5 yrs) were vaccinated against tetanus toxoid (Super-Tet with Havlogen; Intervet Inc., Millsboro, DE). Blood samples were collected via jugular venipuncture for the determination of tetanus specific antibody titers immediately prior to vaccination (d 0) and on d 7, 14, 21, and 28 post-vaccination. Blood was allowed to clot for 10-15 min after collection and then centrifuged at 2500 x *g*. Serum was stored at -80 ºC until further analysis (Saul et al., 2012).

A commercial double antigen tetanus ELISA previously validated for use in humans was used in this study (Bethyl Laboratories; Montgomery, Texas). In each of six separate trials, a 96-well microtiter plates was coated with 0.01 µg tetanus antigen in 50 µl carbonate buffer (pH 9.6) per well to bind the primary antigen to the plate. The plate was incubated overnight at 4°C. A blocking solution of phosphate buffer saline (PBS)/bovine serum albumin (BSA) 1% 50 µl per well was added and incubated overnight at 4°C to ensure that all antigens in the wells were bound to the plate walls and that other solutions

can bind only to the bound antigen. Blocking solution was removed and the plate was left at room temperature for 2 hours. Serum samples diluted to 1:1000 in dilution buffer were added 50 µl per well and incubated for one hour to adhere the antibodies in the serum to the antigen bound to the plate. The plate was then washed to remove unbound antibody. The labeled secondary antigen was then added, 100 µl per well, after being diluted 1:2800, and plates were incubated for one hour. Conjugate Streptavidin, peroxidase-labeled, was then added 100 µl per well, diluted 1:1000 in dilution buffer. This was incubated for one hour at 37°C followed by 50 µl of tetramethyl-benzidine (TMB) added to each well. The plate was placed in a dark room for 10 minutes at room temperature so that tagged secondary antigens could react with the color conjugate. The reaction was terminated by adding 50 µl per well of 1.25 M H_2SO_4 and the absorbances were read on a spectrophotometer at 450nm. Between each step each, the wells were washed three times in PBS pH 7.2 containing 1% Triton X-100.

Results

In order to determine IgG values using the double antigen tetanus ELISA, a standard curve must be generated to extrapolate the IgG values of the unknown serum samples. Based on the known standards used for this assay, the standard curve should have ranged from 15.625 ng/ml to 1000 ng/ml. In each of the six separate trials, a standard curve could not be produced. Two of the plates had optical density values less than 0.09 while three of the plates had optical density values greater than 3.0 (data not shown). In the one trial that did produce optical density values within the expected range (0.13 – 2.328), a standard curve could not be applied (Figure 1). As a result, predicted IgG values from the unknown serum samples could not be consistently produced (Figure 2).

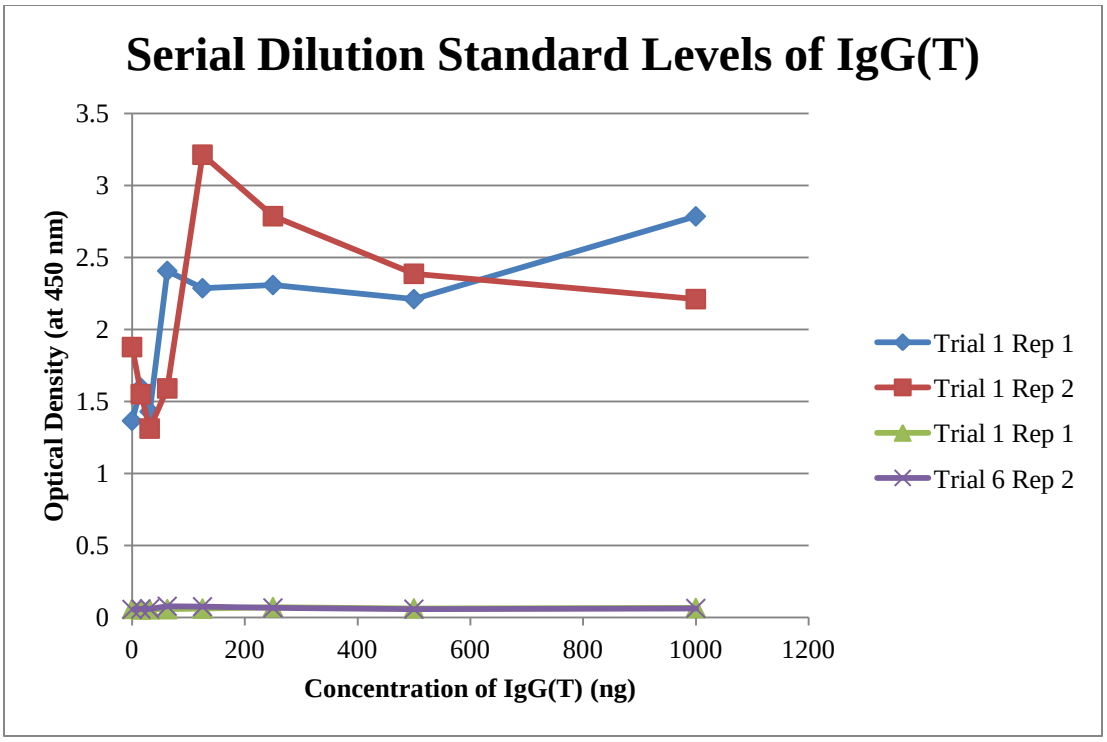

Figure 1. The standard curve generated in two separate trials using the double antigen tetanus ELISA.

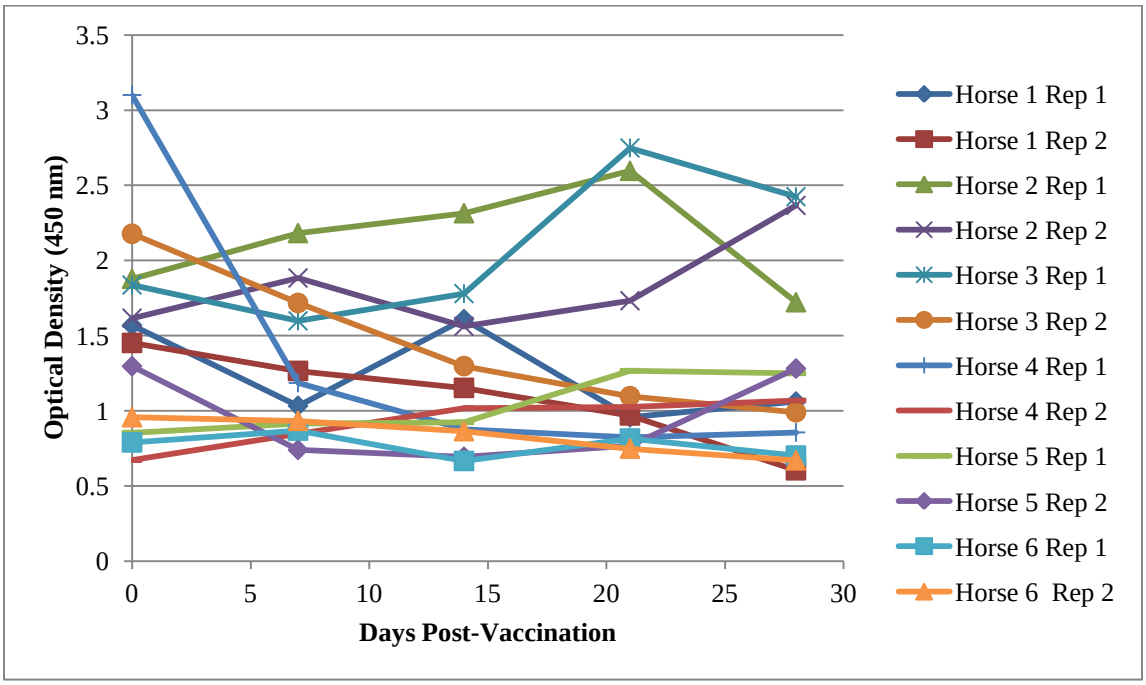

Figure 2. Optical density values generated in one of six separate trials to measure tetanus-specific IgG titers in response to tetanus vacccination.

Discussion

The goal of this research project was to develop a timely, cost-effective and accurate ELISA to quantify tetanus immune responses in horses. A double antigen tetanus ELISA previously validated for use in humans was modified for this purpose. Creating a reliable standard curve with proved to be difficult. Originally, a series of dilutions of the labeled antigen was used as the known standard from which to generate a standard curve. After the initial attempts to generate the standard curve were unsuccessful, questions were raised about the effectiveness of the blocking solution and the antibody-antigen binding as possible causes of the inconsistencies in optical density values for the known standards. The wells in the microtiter plates used in this study held a total volume of 300 μl; however, initially only 100 μl of blocking solution was used. The procedure was adjusted to 300 μl of blocking solution per well with washing three times between each step to ensure that the entire well surface was properly blocked to prevent binding of any proteins to the plate. Although the assay was successful in producing optical density values within the expected range in one of the six trials, a standard curve could not be applied to the values. In addition, these optical density values were not consistent between replicates of the same sample within a trial.

Inconsistent readings from the plates may also have to do with an antigen-antibody binding issue. In a previous double antigen ELISA study in sheep, there was no evidence of cross reactivity between species and bacteria (Rosskopf et al., 2005). This implies that the human antigen used with equine sera samples may not have been close enough in reactivity for a functional ELISA. The tetanus toxin was used as the primary antigen and the labeled secondary was a human tetanus toxin T cell epitope. The antibodies in the equine

serum may not have been able to recognize this as a tetanus antigen due to binding differences between species.

With regard to tetanus specific antigens, previous research has demonstrated that the double antigen ELISA was more specific than an indirect ELISA, and had very comparable results to *in vivo* tests as well as a Toxin Binding Inhibition (ToBI) test (Perry et al., 2009). A double antigen time-resolved fluorescence immunoassay (DELFIA) has also been shown to detect both tetanus and diphtheria antibodies in human serum (Aggerbeck et al., 1996). The DELFIA was able to compare multiple species on the same plate. Kristiansen et al. (1997) found the double antigen method to be specific for tetanus IgG but lacked the ability of the DELFIA to compare species. While the double antigen tetanus ELISA has been shown to be sensitive and reliable with human sera, further modifications of the protocol are needed to improve the reliability with horse serum.

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